AN INTERMEDIATE IN THE BIOSYNTHESIS OF POLYPHENYLALANINE DIRECTED BY SYNTHETIC TEMPLATE RNA

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We have recently found that simple, synthetically prepared polyribonucleotides such as polyuridylic† acid and polycytidylic acid function as RNA templates in a cell-free protein-synthesizing system prepared from *E. coli*.[1, 2]   In this system, poly U contains the information for the synthesis of polyphenylalanine; therefore, the code for phenylalanine is one or more uridylic acid residues.   Poly U was much more effective in increasing the rate of cell-free protein synthesis than naturally occurring informational RNA,[2] possibly because the synthesis of a protein containing only one amino acid was faster than the synthesis of a protein containing 20 amino acids.   We are using this model system currently to study the enzymatic sequence of protein synthesis.

Although sRNA and amino acid–activating enzymes have been studied extensively, there is controversy concerning their relationship to protein synthesis.[3–6]

One purpose of this investigation was to determine whether sRNA is an intermediate in the synthesis of polyphenylalanine directed by a synthetic RNA template.   Our results demonstrate that the incorporation of phenylalanine into sRNA and its subsequent transfer from sRNA are steps in the synthesis of polyphenylalanine.

Methods.—Dialyzed extracts of *E. coli* containing ribosomes and 100,000 × *g* supernatant solutions were obtained as described perviously.[2] These extracts correspond to the previously described S-30 fractions.[2] Ribosomes were sedimented by centrifuging S-30 extracts at 105,000 × *g* for 2 hr at 3°. The supernatant solution was aspirated and will be referred to hereafter as 100,000 × *g* supernatant solution. The ribosomes were resuspended in 0.01 *M* Tris (hydroxymethyl)aminomethane, pH 7.8, 0.01 *M* magnesium acetate, 0.06 *M* KCl, and were centrifuged again at 105,000 × *g* for 2 hr. The supernatant solution was decanted and was discarded. The ribosomes were washed two more times in the same manner by resuspension and centrifugation. Both 100,000 × *g* supernatant solutions and washed ribosomes were stored in small aliquots under liquid nitrogen. Thawed preparations were not refrozen and reused.

sRNA was purified from *E. coli* 100,000 × *g* supernatant solutions by phenol extraction. sRNA was charged with C^{14}-phenylalanine enzymatically, and the C^{14}-aminoacyl-sRNA was purified by the method described by von Ehrenstein and Lipmann.[4] The specific radioactivity of the C^{14}-aminoacyl-sRNA varied from 800 to 36,000 counts/min/mg sRNA. The optical density of 1 mg of sRNA in H_2O at 260 mμ was assumed to be 24.[4]

The RNAase-digested aminoacyl-sRNA described in Table 2 was prepared by incubating C^{14}-phenylalanine-sRNA with 1 μg crystalline RNAase (Worthington Biochemical Corporation) per ml at 37° for 1 hr. The RNAase was removed by 5 consecutive phenol extractions using equal volumes of phenol. The aqueous phase was then dialyzed overnight against 1,000 volumes of H_2O.

The alkali-degraded C^{14}-phenylalanine-sRNA described in Table 2 was prepared by incubating aminoacyl-sRNA in 0.3 *M* KOH at 35° for 18 hr. The solution was then neutralized and was dialyzed against 1,000 volumes of H_2O. Also, C^{14}-phenylalanine-sRNA was incubated in 0.4 *M* glycine buffers, pH 11.0 at 37° for 1 hr. The solution was then dialyzed against 1,000 volumes of H_2O.

Radioactive protein precipitates were washed and counted as before[2] by a modification of the method of Siekevitz.[7] Protein concentrations were determined by a micro modification of the method of Lowry.[8] All assays reported in this paper were performed in duplicate.

Materials.—U-C^{14}-L-phenylalanine was obtained from the Nuclear-Chicago Company and had a specific radioactivity of 5–10 mC/mmole. The purified transfer enzyme (after DEAE column chromatography, purified about 10-fold[9]) and some C^{14}-phenylalanine-sRNA used were the generous gift of Daniel Nathans and Fritz Lipmann. Polynucleotide phosphorylase was used to synthesize poly U. The molecular weight of the poly U was between 30,000 and 50,000.

Results.—Components of reaction mixtures are given in Table 1.   The data presented in Table 1, Experiment 1, demonstrate that C^{14}-phenylalanine was incorporated into protein only when poly U was added to the reaction mixture.   When 2 μmoles of unlabeled, C^{12}-phenylalanine were added to a reaction mixture containing 0.019 μmoles of C^{14}-phenylalanine, the incorporation of C^{14}-phenylalanine into protein was markedly reduced as was expected, due to dilution of the tracer.

C^{14}-phenylalanine-sRNA was prepared by incubating sRNA with C^{14}-phenylalanine and nonpurified phenylalanine activating enzyme present in 100,000 × *g* supernatant solutions. These extracts catalyzed a phenylalanine-dependent exchange of labeled pyrophosphate into ATP. Other amino acid–activating enzymes, stimulating a similar exchange, were also present.

In Experiment 2, the tracer present in reaction mixtures was C^{14}-phenylalanine-sRNA in place of free C^{14}-phenylalanine. ATP, CTP, and UTP were omitted. In the absence of poly U, little C^{14}-phenylalanine was incoporated into protein. However, in the presence of poly U, approximately 60 per cent of the C^{14}-phenyl-

TABLE 1

EFFECT OF C^{12}-PHENYLALANINE UPON C^{14}-PHENYLALANINE INCORPORATION INTO PROTEIN

Experiment No.	C^{14}-tracer	Addition	Counts/min
1	C^{14}-L-Phenylalanine	None	1,430
		− Polyuridylic acid	68
		+ 2 μmoles C^{12}-L-phenylalanine	93
		− ATP, PEP, and PEP kinase	62
		None, zero time	49
2	C^{14}-L-Phenylalanine-sRNA	None	683
		− Polyuridylic acid	9
		+ 2 μmoles C^{12}-L-phenylalanine	603
		− GTP, PEP, and PEP kinase	10
		None, zero time	6

The reaction mixtures for Experiment 1 contained the following in μmoles/ml: 100 Tris (hydroxymethyl)-aminomethane, pH 7.8; 10 magnesium acetate; 50 KCl; 6 mercaptoethanol; 1 ATP; 5 phosphoenolpyruvate, K salt; 2 μg phosphoenolpyruvate kinase, crystalline; 0.03 each of GTP, CTP, and UTP; 10 μg polyuridylic acid; 0.019 μmoles of C^{14}-L-phenylalanine, ~75,000 counts/min; and 0.46 and 1.04 mg of ribosomal and 100,000 × g supernatant solution protein, respectively.

The reaction mixtures for Experiment 2 contained the following in μmoles/ml: 100 Tris (hydroxymethyl)-aminomethane, pH 7.8; 10 magnesium acetate; 50 KCl; 6 mercaptoethanol; 0.3 GTP; 5 phosphoenolpyruvate, K salt; 2 μg phosphoenolpyruvate kinase, crystalline; 10 μg polyuridylic acid; 0.45 mg C^{14}-phenylalanine-sRNA, ~1100 counts/min; and 0.46 and 1.04 mg of ribosomal and 100,000 × g supernatant solution protein, respectively.

Total volume was 0.5 ml. Samples were incubated at 35° for 10 min, were deproteinized with 10 per cent trichloroacetic acid.

alanine was transferred from sRNA to protein. The transfer required GTP and a GTP-generating system; however, with this dialyzed but unpurified system, ATP and an ATP-generating system was 70 per cent as effective as GTP in facilitating transfer. Addition of 2 μmoles of unlabeled C^{12}-L-phenylalanine did not decrease appreciably the transfer of C^{14}-phenylalanine from sRNA to ribosomal protein, in contrast to the data of Experiment 1. These results demonstrate that C^{14}-phenylalanine can be transferred undiluted from sRNA to protein in the presence of a large pool of unlabeled, free phenylalanine and that poly U directs the transfer.

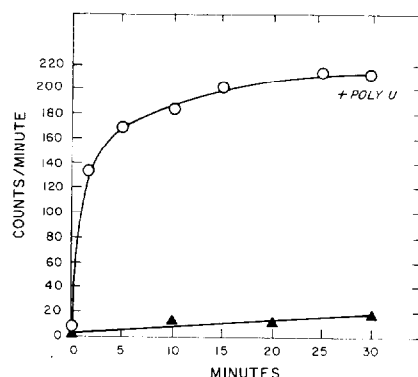In Figure 1, the quantity of phenylalanine transferred from sRNA into ribosomal

FIG. 1.—Stimulation of C^{14}-phenylalanine transfer from sRNA to protein by polyuridylic acid ○ 5 μg polyuridylic acid added; ▲ without polyuridylic acid. The components of the reaction mixtures are given in Table 1, Experiment 2. 0.17 mg of C^{14}-phenylalanine-sRNA. 400 counts/min, 0.46 mg ribosomal protein and 1.04 mg 100,000 × g supernatant fraction protein were added to each reaction mixture.

protein is plotted against time in minutes. In the absence of poly U, little C^{14}-phenylalanine was incorporated. In the presence of poly U, C^{14}-phenylalanine was transferred rapidly from sRNA to protein. Almost all of the C^{14}-phenylalanine was transferred during the first five minutes of incubation.

Some control experiments are presented in Table 2 demonstrating that C^{14}-

TABLE 2

AMINOACYL RNA CONTROL EXPERIMENTS

Additions	Counts/min
C^{14}-phenylalanine-sRNA	704
C^{14}-phenylalanine-sRNA treated with RNAase*	2
C^{14}-phenylalanine-sRNA treated with 0.3 M KOH+	9
C^{14}-phenylalanine incubated at pH 11†	18
None, Zero time	4

The composition of the reaction mixtures is presented in Table 1, Experiment 2. 1.04 mg protein in 100,000 × g supernatant solution, 0.46 mg ribosomal protein, and 0.45 mg C^{14}-phenylalanine sRNA containing ~1100 counts/min (before digestion) were added to each reaction mixture.
* sRNA preparations were deproteinized by phenol extraction after RNAase treatment as specified in the *Methods* section.
† Alkali treatment of C^{14}-phenylalanine-sRNA and incubation at pH 11 are described in the *Methods* section.

phenylalanine-sRNA has properties similar to those reported for aminoacyl-RNA. Treatment of C^{14}-phenylalanine-sRNA with RNAase or with 0.3 N KOH destroyed its activity. Aminoacyl-sRNA is hydrolyzed to free amino acids and sRNA at pH 10.[10] Incubation of C^{14}-phenyl-alanine-sRNA at pH 11 resulted in the hydrolysis of phenylalanine from sRNA as expected. In addition, our preparations of C^{14}-phenyl-alanine-sRNA had a sedimentation value of 4.6 as determined by sucrose density-gradient centrifugation.[11]

The data of Table 3 demonstrate that both 100,000 × g supernatant fractions

TABLE 3

REQUIREMENT FOR 100,000 × g SUPERNATANT SOLUTION AND RIBOSOMES

Additions	Counts/min
None	341
−100,000 × g supernatant solution	14
−Ribosomes	5
None, zero time	7

The components of the reaction mixtures are presented in Table 1, Experiment 2. 0.46 and 1.04 mg protein were present in the ribosome and 105,000 × g supernatant fractions, respectively. 1.0 mg of C^{14}-phenylalanine-sRNA, ~800 counts/min, were added to each sample.

and ribosomes were required for transfer of C^{14}-phenylalanine from sRNA to protein. Since the transfer enzyme is found in 100,000 × g supernatant solutions, it seemed likely that the requirement for this fraction could be replaced by purified transfer enzyme. The data of Table 4, Experiment 1, demonstrate that transfer enzyme

TABLE 4

REPLACEMENT OF 100,000 × g SUPERNATANT FRACTION WITH TRANSFER ENZYME

Experiment No.	Addition	Counts/min
1	None	404
	− Transfer enzyme	10
	− Polyuridylic acid	11
	None, zero time	6
2	+ GTP, + PEP, + PEP Kinase	566
	− GTP, + PEP, + PEP Kinase	17
	− GTP, + ATP, + PEP, + PEP Kinase	68
	+ GTP, + ATP, + PEP, + PEP Kinase	271
	+ GTP, − PEP, − PEP Kinase	17
	+ GTP, + ATP, − PEP, − PEP Kinase	13
	+ GTP, + PEP, + PEP Kinase, Zero Time	3

Components of the reaction mixtures are presented in Table 1, Experiment 2. 100,000 × g supernatant solution was omitted. 2.0 μmoles of ATP/ml of reaction mixture were present where specified. 0.46 mg of ribosomal protein were present and 0.095 mg transfer enzyme protein were present except where specified. 0.30 mg C^{14}-phenylalanine-sRNA, ~800 counts/min, were added to each sample in Experiment 1 and 0.05 mg, ~1300 counts/min, were added to each sample in Experiment 2. In Experiment 2, for simplicity, the presence or absence of GTP, ATP, PEP, and PEP kinase are noted.

could replace $100,000 \times g$ supernatant solution. The data of Experiment 2 show that GTP was necessary for the transfer, and that, in this purified system, ATP could not replace GTP effectively. The data of Tables 1, 3, and 4 show that C[14]-phenylalanine transfer from sRNA to protein required ribosomes, transfer enzyme, poly U, and GTP and a GTP-generating system.

Discussion.—The data presented in this communication demonstrate that aminoacyl-sRNA is an intermediate in phenylalanine incoporation into protein mediated by poly U. In a previous communication, we showed that the protein synthesized had unusual characteristics similar to authentic polyphenylalanine.[2] The initial steps in polyphenylalanine synthesis appear to be:

$$\text{L-phenylalanine} + \text{ATP} \underset{\text{enzyme}}{\overset{\substack{\text{phenylalanine-}\\\text{activating}}}{\rightleftharpoons}} \text{AMP-phenylalanine} + \text{P-P}. \qquad (1)$$

$$\text{AMP-phenylalanine} + \text{sRNA} \underset{\text{enzyme}}{\overset{\substack{\text{phenylalanine-}\\\text{activating}}}{\rightleftharpoons}} \text{phenylalanine-sRNA} + \text{AMP}. \qquad (2)$$

$$\text{Phenylalanine-sRNA} \overset{\substack{\text{Poly U}\\\text{ribosomes}\\\text{transfer}\\\text{enzyme}\\\text{GTP}\\ ?\quad ?\quad ?}}{\rightarrow}\rightarrow\rightarrow \text{polyphenylalanine} + \text{sRNA}. \qquad (3)$$
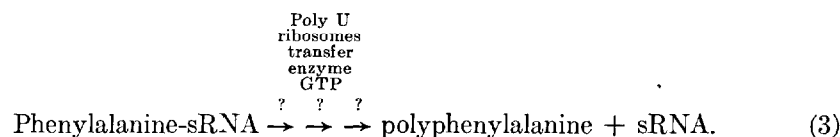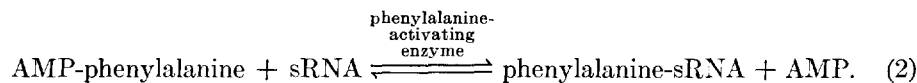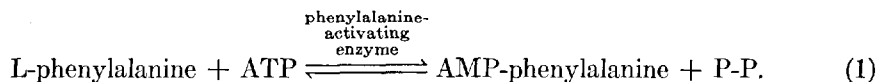
The detailed mechanisms of the steps involved in reaction (3) are under investigation. It should be noted that our data do not preclude the possibility of alternative routes of synthesis of polyphenylalanine.

When poly A and poly U are mixed, doubly- and triply-stranded RNA is formed (U-A and U-U-A).[12, 13] We have shown previously that poly U in the doubly- and triply-stranded state was completely inactive as template RNA.[2] Further experiments have corroborated and extended these findings and will be published in a separate communication. These data strongly suggest that the portion of the RNA molecule which functions as a template for protein synthesis is single-stranded. Simple predictions may be made concerning the primary and secondary structures of the hypothetical "template-recognition portion" of phenylalanine-sRNA. Since a sequence of one or more uridylic acid residues in poly U is the code for phenylalanine in this system, it is probable that phenylalanine-sRNA contains a complementary sequence of one or more adenylic acid residues which base-pair with the template. It is also probable that the portion of sRNA recognizing the template is single-stranded.

The genetic code may not be universal; it may differ from species to species. Since sRNA may be a cofactor which functions as an "adaptor" carrying an amino acid to its proper place on template RNA, a variant sRNA base-pairing with a *different* code letter of template RNA would substitute one amino acid for another during protein synthesis.[14] Although *E. coli* sRNA can be used for the cell-free synthesis of rabbit hemoglobin,[3, 4] it is possible that in species other than *E. coli*, poly U may be either meaningless or may serve as a template for a different amino acid.‡ Changes in the code could occur at different stages in the translation of information from DNA to the finished protein, for example, at the level of the DNA or RNA templates, at the level of sRNA, or at the level of amino acid–activating

enzymes.   We are using the poly U system to determine whether the code for phenylalanine is the same in different species.

Summary. —Phenylalanine-soluble RNA was shown to be an intermediate in the cell-free synthesis of polyphenylalanine directed by a synthetic template RNA, polyuridylic acid.

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† The following abbreviations are used: Polyuridylic acid, poly U;   polycytidylic acid, poly C;   RNA, ribonucleic acid;   DNA, deoxyribonucleic acid;   sRNA, soluble ribonucleic acid;   RNAase, ribonuclease;   ATP, adenosine triphosphate;   UTP, uridine triphosphate;   CTP, cytidine triphosphate;   and GTP, guanosine triphosphate;   PEP, phosphoenolpyruvate;   and PEP kinase, phosphoenolpyruvate kinase.

‡ That this may be the case is suggested by preliminary experiments performed in collaboration with Harry Gelboin showing that poly U does not stimulate incorporation of C^{14}-phenylalanine in a rat liver amino acid–incorporating system.   Similar results have been obtained by S. Ochoa (personal communication).

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